Early host cell reactivation of an oxidatively damaged adenovirus-encoded reporter gene requires the Cockayne syndrome proteins CSA and CSB

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Reduced host cell reactivation (HCR) of a reporter gene containing 8-oxoguanine (8-oxoG) lesions in Cockayne syndrome (CS) fibroblasts has previously been attributed to increased 8-oxoG-mediated inhibition of transcription resulting from a deficiency in repair. This interpretation has been challenged by a report suggesting reduced expression from an 8-oxoG containing reporter gene occurs in all cells by a mechanism involving gene inactivation by 8-oxoG DNA glycosylase and this inactivation is strongly enhanced in the absence of the CS group B (CSB) protein. The observation of reduced gene expression in the absence of CSB protein led to speculation that decreased HCR in CS cells results from enhanced gene expression in the absence of CSB protein. The interpretation of this enhanced expression of the damaged gene requires the Cockayne syndrome proteins CSA and CSB.

Introduction

The correct chemical structure of DNA bases allows the specific pairing of adenine with thymine and guanine with cytosine, forming the basis by which accurate transmission of hereditary information through DNA replication and functional information through transcription occurs. The chemical properties of the bases leave them susceptible to alteration by a number of factors including reactive oxygen species (ROS) generated endogenously by metabolic processes of the cell, from environmental sources including ultraviolet (UV) radiation, ionising radiation and dietary sources. Due to its low redox potential, guanine is particularly susceptible to oxidation by ROS and can form a large number of oxidised products (1). Of the many oxidised guanine products formed, 7,8-dihydro-8-oxoguanine (8-oxoguanine; 8-oxoG) is one of the most frequent forms of oxidative DNA lesions generated in living cells (2). 8-oxoG can functionally mimic T, allowing it to easily base pair with either A or C during replication and transcription. Failure to recognise and repair an improper 8-oxoG:A following replication results in G to T transversions during replication and mutant transcripts during transcription of nascent mRNAs (1). The base excision repair (BER) pathway is responsible for removing oxidised bases, including 8-oxoG from chromosomal DNA and relies on DNA glycosylases to recognise specific lesions and initiate the repair process (3). 8-oxoguanine DNA glycosylase (hOGG1 in humans; OGG1 in mice) recognises 8-oxoG lesions base-paired with C and catalyses excision of the damaged base, initiating its repair (for a review of BER, see ref. 3). In addition, the Cockayne syndrome group B protein (CSB) has been shown to be involved in processing of the lesion (4–6). Among the many functions of CSB, the best studied to date is the role it plays in conjunction with the Cockayne syndrome group A (CSA) protein in coupling transcription by RNA polymerase II (polII) to repair of nucleotide excision repair (NER) substrates in actively transcribed genes via the transcription-coupled repair (TCR) subpathway of NER (7). Mutations in CSA and CSB in humans normally causes Cockayne syndrome, a segmental progeroid syndrome characterised by premature ageing and neurodegeneration beginning in early childhood (8). Using a plasmid-based β-galactosidase (β-gal) reporter gene assay, we have examined the effect of methylene blue plus visible light (MB+VL)-induced 8-oxoG lesions on the time course of gene expression in normal and CSA and CSB mutant human SV40-transformed fibroblasts, repair proficient and CSB mutant Chinese hamster ovary (CHO) cells and normal mouse embryo fibroblasts. We demonstrate that MB+VL treatment of the reporter leads to reduced expression of the damaged β-gal reporter relative to control at early time points following infection in all cells, consistent with in vivo inhibition of RNA polIII-mediated transcription. In addition, we have demonstrated HCR of reporter gene expression occurs in all cell types examined. A significant reduction in the rate of gene reactivation in human SV40-transformed cells lacking functional CSA or CSB compared to normal cells was found. Similarly, a significant reduction in the rate of reactivation in CHO cells lacking functional CSB (CHO-UV61) was observed compared to the wild-type parental counterpart (CHO-AA8). The data presented demonstrate that expression of an oxidatively damaged reporter gene is reactivated over time and that CSA and CSB are required for normal reactivation.
spontaneously transformed mouse embryoblasts (MEFs) between 8 and 48 h after transfection. Compared to the non-
damaged control vector, the 8-oxoG containing reporter
demonstrated the same level of transcription at 8 h with
~50% reduction in transcription over a subsequent 40-h period
(10). In addition to wild-type (WT) MEFs, reporter gene
expression was also examined in Csbrm, Ogg1+/− and Csbrm
Ogg1−/− immortised MEFs. The authors suggest that Ogg1
mediates inactivation of the reporter gene and that the effect
is considerably enhanced by the absence of functional CSB (10).
Based on their finding of no difference in reporter gene
expression from damaged and undamaged plasmids at 8 h after
infection, Khobta et al. (10) also conclude that 8-oxoG does
not impede RNA polII. In addition, they conclude that the
decreased expression levels of the oxidatively damaged
reporter gene in CSB-deficient cells compared to normal cells
(9,11) are not due to the lack of CSB-mediated repair/
reactivation of the reporter construct, but rather Ogg1/hOGG1
mediated inactivation that is enhanced in the absence of
functional CSB. Recently presented data by the same group
(12) demonstrated decreased acetylation of histone H4 in
oxidatively damaged plasmid DNA introduced into HeLa cells,
suggesting gene silencing may be mediated by chromatin
alterations in the 8- to 48-h period following transfection of the
plasmid.

To further study the effect of 8-oxoG on gene expression, we
have examined expression of a recombinant adenovirus-encoded
β-gal reporter gene damaged by MB + VL in normal
and CSA and CSB mutant SV40-transformed human fibro-
blasts; WT MEFs and repair-proficient and repair-deficient
CHO cells with a mutation in the hamster CSB homologue. We
demonstrate that MB + VL treatment of the reporter gene leads
to reduced expression relative to control at early time points
following infection in all cells examined and that HCR of
reporter gene expression occurs in all cell types examined by
a mechanism requiring CSA and CSB.

Materials and methods

Cells, virus and culture conditions
The SV40-transformed fibroblasts GM637F (normal), CSA-SV40
(CS3BE.S3.G1) and CSB-SV40 (CS1AN.S3.G2) were obtained from NIGMS
(Camden, NJ, USA). The spontaneously transformed MEF line WT MEF
(ERRCC111; referred to as WT MEF hereafter) (13) was obtained from Dr D-X
Zhu McMaster University, Hamilton, Ontario, Canada. The SV40-transformed
mouse embryoblast cell line BCl-6 was from Dr S.E. Andrew University
of Alberta, Edmonton, Alberta and has been previously described (14). The
Chinese hamster ovary (CHO) cell lines CHO-AA8 (repair proficient parental)
and CHO-UV61 (repair deficient) were provided by Dr Larry Thompson,
Lawrence Livermore National Laboratory, Livermore, CA with the help of
Dr Gordon Whitmore, Physics Division, Ontario Cancer Institute, Toronto,
Ontario. Cell cultures were grown at 37°C in a humidified incubator in 5% CO2,
and cultured in Eagle’s χ-minimal essential media (χ-MEM) supplemented
with 10% foetal bovine serum and antimycotic antibiotic (100 μg/ml penicillin,
100 μg/ml streptomycin and 250 ng/ml amphotericin B). The recombinant
adenoviruses Ad5CMVlacZ (AdCA17) and Ad5CMVlacZ (AdCA35) were
obtained from The Robert E. Fitzhenry Vector Laboratory, McMaster
University, Hamilton, Ontario. The viruses were propagated, collected and
titered as described previously (15).

Treatment of the virus with MB + VL
Preparation of MB was carried out under minimal ambient light conditions as
described previously (16). Treatment of the virus was performed under minimal
ambient light conditions as described previously (17). Briefly, a 4 ml volume of
20 μg/ml (53.5 μM) MB solution was prepared by diluting the MB stock in
cold phosphate-buffered saline (PBS) (4°C) in a 35-mm Petri dish and
subsequently shielded from any ambient light. An appropriate volume of stock
Ad5CMVlacZ was added to the Petri dish containing the MB solution in order
to obtain the desired multiplicity of infection (MOI) upon infecting cells.
Prior to exposing the virus to VL, an aliquot was removed for use as the
undamaged control. The solution was then exposed to VL using a 1000 W bulb
(Genral Electric, GE R1000) at a distance of 82 cm from the source for a
defined period of time while being kept on ice with continuous stirring. After
each defined VL exposure, aliquots were removed for use in infecting cells. The
aliquots were diluted in unsupplemented χ-MEM and used to infect cells.

Treatment of the virus with UV
UV irradiation of the virus was carried out as previously described (18).
Briefly, the virus was resuspended in a 35-mm Petri dish in cold PBS at the
appropriate dilution to achieve an MOI of 100 pfu/ml upon infection of cells.
Using a General Electric germicidal lamp (model GST5) emitting pre-
dominantly at 254 nm, the virus was irradiated with stirring on ice with an
incident fluence rate of 2 j/m2/sec. After each UV exposure, 200 μl aliquots
were removed from the viral preparation and appropriately diluted using
unsupplemented χ-MEM.

β-Galactosidase reporter gene expression assay (HCR)
We have previously reported a HCR assay for examining BER of MB + VL-
induced 8-oxoG lesions in a number of different cell strains (16,17,19). The
HCR assay utilises a recombinant non-replicating adenovirus (Ad) expressing
the β-gal reporter gene under control of (16,17,19) the murine cytomegalovirus
immediate early promoter (20) to examine the ability of different cell types to
remove damage and reactivate reporter gene expression. Upon exposure to VL,
MB leads to the formation of 8-oxoG lesions with a small number of other
single-base oxidative lesions occurring (21,22). Cells were seeded for
confluence (SV40-transformed cells at 3.5 × 105 cells per well; MEFs and
CHO cells at 4 × 105 cells per well) in 96-well plates (Falcon, Franklin Lakes,
NJ, USA). After seeding, cells were incubated for 18–24 h and subsequently
infected with 40 μl of untreated or MB + VL-treated virus for 90 min at a MOI
of 100 pfu/cell. Following the 90-min viral absorption period, the infection
medium was aspirated and cells were overlaid with 200 ml of complete χ-MEM
and incubated for a further 1, 2, 3, 6, 12, 24 or 44 h before harvesting for
measurement of β-gal activity. For the time course experiments, cells from
the same pool were seeded into separate 96-well plates for each time point
and infected with virus from the same preparation. A single HCR experiment
consisted of triplicate wells for each treatment of the virus and triplicate wells
of non-infected cells were used to obtain background levels of β-gal activity.
β-Gal activity was scored as previously described (23).

Graphing and statistical analysis
All curves for inactivation of β-gal activity following MB + VL treatment
of the virus were plotted using Origin Laboratory software. Each point on the
graphs represents an arithmetic mean ± standard error of triplicate
determinations of the β-gal activity at each VL exposure to the virus relative
to the untreated control for single representative experiments. D37 values for
each cell line were obtained by extrapolation from the HCR survival curves
from each independent experiment. D37 values for each cell type were then
calculated relative to the indicated repair-proficient cell line used within the
same experiment and used as a measure of relative HCR capacity. For
individual time course experiments, the D37 values for each cell line at each
time point were calculated relative to the indicated repair proficient normal
at 12 h after infection. The data calculated in this manner were pooled from
independent experiments to construct the D37 reactivation time course curves.
Statistical analysis of differences between relative D37 values was carried out
using a one-sample two-tailed t-test with a confidence interval of 0.05. The χ2
goodness of fit test was used to quantify how well the D37 reactivation time
course curves for repair-deficient cell lines matched the curves for the
appropriate repair-proficient control cell line over the range of time points
examined (24). For comparison of two cell types, χ2 values were obtained for
each time point and summed over the indicated ranges to obtain P-values
representing the goodness of fit between the two D37 reactivation curves.

Results
Normal HCR of the MB + VL-damaged reporter gene in SV40-
transformed human skin fibroblasts requires CSA and CSB
In the present work, we first examined expression from a
MB + VL-damaged reporter gene using our recombinant
adenovirus-based β-gal gene. In agreement with the results of
Spivak and Hanawalt (9) using a plasmid-based reporter gene,
we find a significant deficiency in reporter gene expression
from CSA-SV40 and CSB-SV40 fibroblasts compared to the normal GM637F fibroblasts 44 h following infection with the MB + VL-treated adenovirus (Figure 1). Similar results were obtained at 24 h following infection with the MB + VL-treated adenovirus (data not shown). To investigate whether reactivation of reporter gene expression was taking place, we examined the expression of the MB + VL-treated reporter gene at various times after infection with the MB + VL-treated adenovirus. To facilitate examining expression at very early time points following infection, we used the AdCA35 virus, which drives higher levels of transgene expression than AdCA17 (20). The expression of the MB + VL-treated reporter gene was reduced compared to the non-treated reporter at early times after infection and expression of the damaged reporter gene increased when β-gal expression was assayed at later times (Figure 2A). The $D_{37}$ time course curve (Figure 2B) was plotted using $D_{37}$ values obtained from HCR plots. Expression of the β-gal reporter is reactivated by the host cell reaching a maximum level by 12 h in both normal and CSB-deficient SV40-transformed fibroblasts. The rate of reactivation was significantly reduced in CSA-SV40 and CSB-SV40 compared to GM637F as measured by the $\chi^2$ goodness of fit test ($P < 0.05$).

It has been reported that repair of UV-induced cyclobutane pyrimidine dimers in plasmids results in a time dependent recovery of transcription from damaged genes resulting from the gradual removal of UV-induced transcription blocking lesion (9,10,25,26). In contrast to the levelling off of reactivation by 12 h after infection for the MB + VL-treated reporter gene, infection of cells with a UVC-treated reporter gene resulted in a continual increase in reporter gene expression from 1 to 72 h after infection of GM637F cells (Figure 2C). Expression from the UVC-damaged virus increased at a significantly slower rate in CSA-SV40 and CSB-SV40 ($P < 0.05$ by $\chi^2$ goodness of fit test), consistent with defective TCR–NER and proficient global genome repair–NER in these cells.

Reactivation of expression from a MB + VL-treated β-gal reporter gene in rodent cells

The experiments showing inactivation of a reporter gene containing oxidative DNA damage were carried out using normal and CSB-deficient MEFs (10). To address the possibility of a difference between human and rodent cells, we next examined expression of the MB + VL-damaged adenovirus-based reporter gene in MEFs and CHO cells. Expression of the MB + VL-treated reporter gene was measured in two different MEF lines. It can be seen that expression of the MB + VL-treated reporter gene increased over time reaching a maximum at 12 h in both WT MEF and BC1-6 (Figure 3A and B). Reporter gene expression was found to be significantly increased between 3 and 12 h for both WT MEF ($P = 0.02$) and BC1-6 ($P = 0.009$) by a two-sample t-test. At time points from 12 to 44 h after infection, we observed a slight decrease in the relative $D_{37}$ value consistent with results published for the expression of a plasmid-based reporter gene during this time period (10,12). However, in the current work, the difference in the relative $D_{37}$ value at 24 or 44 h compared to 12 h was not significant.

We also examined the time course of β-gal expression in repair-proficient and repair-deficient CHO cells to determine if the observed reactivation was specific to mice or if it occurred in other rodent species and to examine the role of CSB in gene reactivation. Figure 4A shows typical inactivation curves for β-gal activity in the repair-proficient parental line CHO-AA8 and the repair-deficient line CHO-UV61 carrying a mutation in the hamster homologue of CSB (27) and Figure 4B shows the time course of gene reactivation. These results demonstrate that the expression of the β-gal reporter was reactivated by the host cell in both CHO-AA8 and CHO-UV61 cells. This reactivation was significant by two-sample t-test from 3 to 6 h for CHO-AA8 ($P = 0.009$) and 3 to 12 h in CHO-UV61 ($P = 0.001$). In addition, the increase in reactivation over time (3–44 h) was significantly reduced in the CSB mutant CHO-UV61 compared to CHO-AA8 as determined by the $\chi^2$ goodness of fit test ($P = 0.0439$).

Discussion

The data presented here demonstrate that expression of a reporter gene containing oxidative DNA lesions is reactivated over time (1–12 h) by host cell mechanisms and that a normal level of reactivation in human fibroblasts requires the CSA and CSB gene products. The previous report by Khobta et al. examined the time course of expression from a plasmid-based reporter containing low levels of 8-oxoG in MEFs beginning 8–12 h after introduction through to 48 h. A reduction in gene expression with time was observed in repair-proficient WT MEFs, repair-deficient $Ogg1^{-/-}$ MEFs and to a greater extent in $Csb^{min}$ MEFs expressing a mutated copy of CSB mimicking the mutation in the human patient CS1AN (10,28). Based on their results, they suggested that oxidative DNA damage led to Ogg1/CSB-mediated reporter gene inactivation (10).
In the current work, we show that the change in relative $D_{37}$ for expression of the MB $+$ VL-treated adenovirus-encoded reporter gene slows and levels off from 12 to 44 h; however, no significant differences were observed between pooled relative $D_{37}$ values over that time period. More importantly, compared to early times after infection (1–3 h), expression from the MB $+$ VL-treated reporter was significantly increased at the later time points consistent with gene reactivation.

While the ability of 8-oxoG to block RNA polII transcription has been disputed (29–32), the observation that CSB can improve RNA polII elongation through 8-oxoG lesions suggests that at the very least, 8-oxoG leads to transient stalling of the RNA polII transcription complex (29). We demonstrate here that at early times after infection (1–3 h) with the reporter gene containing MB $+$ VL-induced oxidative damage, gene expression is inhibited compared to the undamaged control in all cell types examined. The decrease in expression from the MB $+$ VL-damaged adenovirus reporter compared to the undamaged control for all VL doses is consistent with an in vivo decrease of RNA polII transcription due to 8-oxoG lesions on the template strand. Reactivation of gene expression is consistent with BER and/or bypass of 8-oxoG lesions.

It has been shown that repair of 8-oxoG in MEF genomic DNA, as measured by loss of formamidopyrimidine-DNA glycosylase-sensitive sites (FSS) is $\sim$50% complete 4 h after induction by a photosensitiser (Ro 19-8022) and VL, $>80\%$ complete by 8 hrs and nearly 100% complete at 16 h (33). In the current work, expression of the MB $+$ VL-damaged reporter gene in MEFs, CHO cells and human fibroblasts was found to be significantly increased between 1 and 12 h, but levelled off by 12 h after infection. This suggests that
reactivation of the reporter gene is near completion by 12 h after infection, consistent with the time course for removal of 8-oxoG lesions from cellular DNA. In contrast, reactivation of the UVC-damaged reporter gene in the GM637F normal human fibroblasts continued to increase from 1 to 72 h after infection, consistent with the longer time course for removal of UVC-induced DNA damage in human cellular DNA (34,35).

Similar to human fibroblasts and MEFs, we observed a significant increase in reporter gene expression relative to control in WT CHO-AA8 cells and mutant CHO-UV61. The results presented are consistent with a role for the CSB homologue in hamsters in repair and/or bypass of MB + VL-induced 8-oxoG. The rate of change in relative $D_{37}$ over time in CHO cells slows between 12 and 24 h; however, the continuous increase in gene reactivation over the time points examined suggests reactivation of MB + VL-induced 8-oxoG continues over a longer period of time in CHO cells compared to MEFs. Hamster cells do indeed repair 8-oxoG, as measured by loss of FSS at a slower rate than mouse cells with only 56%...
of FSS removed at 8 h (36) compared to >80% in mouse cells at the same time point (33). The hamster homologue of CSB, which is involved in repair of UV-induced damage (27) and oxidative damage (36), shows no significant involvement in RNA polII-mediated transcription (27). This suggests that CSB-stimulated lesion bypass and/or coupling of repair to transcription may be absent in hamster cells, possibly accounting for slower repair and subsequent gene reactivation.

It is possible that the decreased level of gene expression seen in MEFs lacking functional CSB compared to WT MEFs, as previously reported (10), could result from a failure of cells lacking CSB to reactivate gene expression from a damaged reporter to WT levels prior to 12 h. In the current work, we were unable to detect a significant change in expression of the MB + VL-treated reporter between 12 and 44 h after infection, whereas Khobta et al. (10) demonstrated a significant decrease in reporter gene expression over this time period. This difference could result from differences in the experimental conditions and the reporter gene employed.

Both studies employed MB + VL to generate 8-oxoG lesions in the reporter gene. MB + VL induces high yields of 8-oxoG, while oxidative pyrimidine modifications, sites of base loss and single-strand breaks (SSBs) are rare (37). The generation of SSBs by MB + VL has been shown to occur at a rate of ~0.1 modifications per 10 000 bp, while 8-oxoG occurs at a rate of 2.7 modifications per 10 000 bp in supercoiled plasmid DNA (37). A similar ratio of SSBs to 8-oxoG lesions was observed in DNA isolated from treated bacteria (37) suggesting the mechanism and profile of DNA damage is independent of the system in which it is treated. Khobta et al. (10) employed MB + VL treatment of covalently closed plasmids of 0.8 μM MB + VL exposures resulting in the induction of an average of three FSS per plasmid, corresponding to an average of ~0.5 FSS per transcribed strand of their GFP-encoding reporter gene. In the present work, we employed 53.5 μM MB plus from 10 to 150 sec VL treatment to the recombinant adenovirus-encoding lacZ reporter gene. Previous reports have shown that treatment of adenovirus with MB + VL has negligible effects on the protein capsid and subsequent infectivity of viral particles (38). Preliminary results indicate that VL exposures to the virus of 10–150 sec would result in an average of about one to six FSS per transcribed strand of the lacZ reporter gene (data not shown). This indicates that the 8-oxoG lesion frequency from VL exposures to the virus of 10–150 sec used in the HCR experiments reported here were somewhat greater those used in the study by Khobta et al., although of a similar order of magnitude.

In the work presented here, we utilised an adenovirus-based reporter system with confluent cultures, whereas Khobta et al. used a plasmid-based system with exponentially growing cultures (10,39). Regardless of the level of DNA damage, p53 upregulation, transcriptional activation and p53-dependant apoptosis are attenuated in confluent cultures compared to sparse growing cultures lacking cell–cell contacts (40). The higher cell density of the confluent cultures used in the current work may more closely reproduce the microenvironment in a living organism and account for the observed difference in gene inactivation. Differences in the level of expression and stability of the adenovirus encoded compared to the plasmid encoded reporter gene may account for the difference in the level of gene inactivation observed. Differences in the degree of chromatin association/modification between the two systems may also contribute to the difference (12).

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